30. (New) A LM609 CDR-grafted light chain polypeptide comprising a light chain polypeptide, or a functional fragment thereof, encoded by a LM609 CDR-grafted light chain variable region nucleotide sequence referenced as SEQ ID NO:3, or a modification thereof.

D7

31. (New) The LM609 CDR-grafted heavy chain polypeptide of claim 30, wherein said functional fragment comprises a variable chain polypeptide or a CDR polypeptide.--

REMARKS

Claims 1-18 are under examination in the application. Claims 1, 3-15, and 17 have been amended above. New claims 26-31 have been added. Support for the amendments and new claims can be found throughout the specification. Specifically, support for the amendment to a heavy chain polypeptide sequence "having greater than 88% identity with" SEQ ID NO:2 and a light chain polypeptide sequence "having greater than 79% identity with" SEQ ID NOS:4 or 32 can be found, for example, on page 45, lines 5-8. Support for the amendment to "a modification thereof that does not change the encoded amino acid sequence" can be found, for example, on page 17, lines 24-26. Support for new claims 26-31 can be found, for example, on page 5, line 26, to page 6, line 7; page 14, line 24, to page 15, line 3; page 17, line 21, to page 18, line 11, and page 18, lines 24-29; and in Figure 1. Accordingly, these amendments and new claims do not raise an issue of new matter and entry thereof is respectfully requested.

Applicant appreciates the time and helpful discussions held with Applicant's representative in the informal interview with Examiner Gambel and Mr. Schwartz on August 3, 1999,.

The present invention provides a LM609 CDR-grafted antibody comprising at least one heavy chain polypeptide comprising a variable region amino acid sequence having greater than 88% identity with that shown in Figure 1A (SEQ ID NO:2) and at least one light chain polypeptide comprising a variable region amino acid sequence having greater than 79% with that shown in Figure 1B (SEQ ID NO:4). The antibodies of the invention are non-mouse antibodies or functional fragments thereof that contain heavy and light chain CDR amino acid sequences derived from LM609 and have integrin $\alpha_v\beta_3$ binding activity, integrin $\alpha_v\beta_3$ binding specificity or integrin $\alpha_v\beta_3$-inhibitory activity. Nucleic acids encoding LM609 CDR-grafted antibody heavy and light chains are additionally provided. Applicant has reviewed the Office Action and respectfully traverse all grounds for rejecting the claims for the reasons that follow.

PROVISIONAL REJECTIONS OVER U.S. APPLICATION SERIAL NO. 08/790,540

Claims 1-18 stand provisionally rejected under the judicially created doctrine of obviousness type double patenting as allegedly unpatentable over claims 1-48 of copending application serial number 08/791,391. Applicant respectfully requests that this provisional ground of rejection be deferred until there is an indication of allowable subject matter.

The Office Action appears to indicate that commonly assigned application serial No. 08/791,391 would form the basis for a rejection of the claims under 35 U.S.C. § 103 if the commonly assigned case qualifies as prior art under 35 U.S.C. §102(f) or (g) and the conflicting inventions were not commonly owned at the time the invention in this application was made. The Office Action states that, in order for the Examiner to resolve this issue, the assignee is required under 37 C.F.R. § 1.78(c) to either show that the conflicting inventions were commonly owned at the time the invention in the instant application was made or to name the prior inventor of the conflicting subject matter. The Office Action states that a failure to comply with this requirement will result in a holding of abandonment of the application. The Office Action concludes that a showing that the inventions were commonly owned at the time the invention in this application was made will preclude a rejection under 35 U.S.C. § 103 based upon the commonly assigned case as a reference under 35 U.S.C. § 102(f) or (g).

To satisfy the request in the Office Action to comply with 37 C.F.R. § 1.78(c), pursuant to 37 C.F.R. § 1.104(a)(5)(i) Applicant submits herewith a copy of the Assignment made in co-pending application serial No. 08/791,391, showing that the application was assigned to Ixsys, Inc. and that the Assignment was recorded at Reel 8480, Frame 0714 (Exhibit 1). Also submitted herewith is a copy of the Assignment made in the above-identified application, showing that the subject application was also assigned to Ixsys, Inc. and that the Assignment was recorded at Reel 8608, Frame 0497 (Exhibit 2). In accordance with the requirements of 37 C.F.R. § 1.104(a)(5)(i), Applicant herewith

makes of record in the above-identified application the Assignment of co-pending application serial No. 08/791,391, which shows that the entire rights have been conveyed to Ixsys, Inc. Applicant respectfully submits that the showing herein of assignment of the entire rights to Ixsys, Inc. of both the above-identified application and co-pending application serial No. 08/791,391 satisfies the requirements of 37 C.F.R. § 1.78(c), pursuant to 37 C.F.R. § 1.104(a)(5)(i) and as set forth in M.P.E.P. 706.02(l), as it relates to evidence required to establish common ownership for rejections under 35 U.S.C. § 103(c) for subject matter that qualifies as prior art only under 35 U.S.C. § 102(f) or (g). Accordingly, Applicant respectfully requests that the requirement for showing that the above-identified application and co-pending application serial No. 08/791,391 were commonly owned be withdrawn.

REJECTIONS UNDER 35 U.S.C. § 112

Claims 1-18 stand rejected under 35 U.S.C. § 112, first and second paragraphs, for the use of the term "substantially the same." Applicant maintains that the term "substantially the same" is clear in view of the teachings in the specification and that the specification provides enablement for a substantially the same amino acid or nucleotide sequence.

Applicant would like to clarify for the record the meaning of the term "substantially the same," as taught in the specification. Applicant respectfully disagrees with the Examiner's assertion in section 16 on page 7, lines 9-11, that

"applicant's specification and arguments, including those filed in Paper No. 5, have indicated that "substantially the same" encompasses LM609 or LM609 grafted antibody" and in section 19 on page 9, lines 9-11, of the Office Action mailed March 18, 1999, that "applicant's specification and arguments, filed in Paper No. 6, also indicate that "substantially the same" encompasses LM609, Vitaxin or LM609 grafted antibody." As taught in the specification on page 12, line 18, to page 13, line 16, and as discussed in the response mailed March 3, 1998, and the response mailed December 9, 1998, a "substantially the same" sequence means a nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence identity when compared to a reference sequence. A "nucleotide sequence which is substantially the same nucleotide sequence as a heavy or light chain of LM609, **or** a LM609 grafted antibody including fragments thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as encoding or as being the amino acid sequence of LM609 **or** a LM609 grafted antibody" (page 13, lines 1-7; emphasis added). An "amino acid sequence which is substantially the same amino acid sequence as a heavy or light chain of LM609 grafted antibody or functional fragment thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as representing the amino acid sequence of a LM609 grafted antibody and minor modifications thereof" (page 13, lines 9-16). Accordingly, a nucleotide or amino acid sequence that is substantially the same as a LM609 grafted antibody is recognizable as a LM609 grafted antibody. A nucleotide sequence that is substantially the same as LM609 is recognizable as LM609. Thus, Applicant respectfully submits that

the conclusion in the Office Action mailed March 18, 1999, that "substantially the same" encompasses LM609 or LM609 grafted antibody is an oversight and inaccurate.

Although Applicant believes that the teachings in the specification regarding the meaning of the term "substantially the same" are sufficient to enable one skilled in the art to practice the invention as claimed and that the term "substantially the same" is clear and definite, in order to further prosecution, Applicant nevertheless has deleted the term "substantially the same" from the claims reciting said term. Therefore, Applicant respectfully submits that this rejection has been rendered moot by the deletion of the term "substantially the same" from the claims and respectfully request that this rejection under 35 U.S.C. § 112, first and second paragraphs, be withdrawn.

REJECTIONS UNDER 35 U.S.C. § 102

An issue of public use or on sale activity has been raised under 35 U.S.C. § 102(b). The Office Action states that articles in Biotechnology Newswatch, dated January 16, 1995, and February 6, 1995, disclose the use of LM609 antibody, including the humanized version of the antibody.

Applicant respectfully maintains that the claimed LM609 CDR-grafted antibody was not in public use or on sale in this country more than one year prior to the filing date of the above-identified application. The lack of on sale or public use

activity was corroborated by evidence presented in the form of a Declaration by Dr. Huse in the previous response mailed December 9, 1998.

In regard to the previously submitted Declaration by Dr. Huse, Applicant respectfully disagrees with the assertion in the Office Action in section 13 on page 5 that "certain nucleic acids and cell lines associated with Vitaxin or the CDR-grafted LM609 antibody were the subject of these agreements." As indicated in the previously filed Declaration, nucleic acids encoding any LM609 grafted antibody, any cell lines containing nucleic acids encoding LM609 grafted antibody, and LM609 grafted antibody were maintained under the control of Ixsys, and confidentiality was maintained in any agreements with third parties. Furthermore, Applicant submits herewith as Exhibit 3 a Rule 132 Declaration by Dr. Huse attesting that Celltech Biologics was under obligation of confidentiality for use of any LM609 grafted antibody, including Vitaxin, nucleic acids encoding any Vitaxin or LM609 grafted antibody, cell lines containing nucleic acids encoding any Vitaxin or LM609 grafted antibody, and any related materials or information. Pages of the agreement between Ixsys and Celltech Biologics, provided as Exhibit A, show that the term "product" refers to LM609 grafted antibody. Exhibit A further shows that any LM609 grafted antibody, including Vitaxin, as well as nucleic acids encoding any Vitaxin or LM609 grafted antibody, cell lines containing nucleic acids encoding any Vitaxin or LM609 grafted antibody, and any related information, is obligated to be maintained in confidence by Celltech Biologics and any third parties involved in testing of the LM609 grafted antibody.

The Declaration states that Ixsys maintained control and confidentiality of Vitaxin and LM609 grafted antibody, nucleic acids encoding Vitaxin and LM609 grafted antibody, cell lines containing nucleic acids encoding Vitaxin and LM609 grafted antibody, related materials and their use during any involvement with third parties. The Declaration further states that neither Vitaxin nor LM609 grafted antibody were on sale or in public use more than one year prior to January 30, 1997.

The Office Action requests that "the role of Scripps as the licensee" be addressed. Applicant respectfully submits that no statements or other information have been made of record indicating that Scripps is a licensee.

In light of the above remarks and evidence in the form of Declarations by Dr. Huse submitted in the previous response mailed December 9, 1998, and herewith as Exhibit 3, Applicant respectfully maintains that LM609 grafted antibody and Vitaxin were neither on sale or in public use in this country more than one year prior to the filing date of the above-identified application and respectfully request that this rejection be withdrawn.

Claims 1-18 stand rejected under 35 U.S.C. § 102(e) as allegedly anticipated by Brooks et al., U.S. Patent 5,753,230, issued May 19, 1998 (Brooks et al., 1998). The Office Action alleges that Brooks et al., 1998, describes LM609 antibody and humanized forms of the antibody as well as methods of using LM609 antibody and humanized forms thereof. The Office Action alleges

that the claimed functional limitations would be inherent properties of the LM609 antibody and humanized forms thereof.

Applicant respectfully submits that the claimed compositions directed to a LM609 CDR-grafted antibody are novel over Brooks et al., 1998. In particular, claims 1, 15 and 17, as amended, are directed to LM609 CDR-grafted antibody and polypeptides comprising a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable region amino acid sequence having greater than 79% identity with SEQ ID NO:4.

In contrast, Brooks et al., 1998, does not teach the claimed human acceptor framework sequences with LM609 CDRs corresponding to SEQ ID NOS:2 and 4 for LM609 CDR-grafted antibody. Therefore, Applicant respectfully submits that Brooks et al., 1998, does not teach the claimed antibodies having the structural characteristics of the specifically recited SEQ ID NOS.

The Office Action alleges that Brooks et al., 1998, describes the LM609 antibody as well as humanized forms of this antibody and claims methods of using the LM609 antibody as well as humanized forms of this antibody. Applicant respectfully submits that Brooks et al., 1998, at most indicates that humanized forms of LM609 may be made. However, Applicant contends that it is well known that CDR grafting often results in loss of antigen binding affinity. As evidence that it is well known that CDR grafting and humanization of antibodies often results in lower activity, Applicant submits herewith as Exhibit

4 a reference by Rader, Cheresh and Barbas, Proc. Natl. Acad. Sci. USA 95:8910-8915 (1998). As indicated on page 8914, column 2, first full paragraph of Rader et al.:

> CDR grafting often yields humanized antibodies with much lower affinity because framework residues are involved in antigen binding, either indirectly, by supporting the conformation of the CDR loops, or directly, by contacting the antigen (26)[Foote and Winter, J. Mol. Biol. 224, 487-499 (1992)]. Therefore, it is usually necessary to replace certain framework residues in addition to CDR grafting. The fact that about 30 framework residues potentially contribute to antigen binding (26)[Foote and Winter, *supra*] makes this fine-tuning step very laborious.

In contrast to Applicant's explicit teachings of methods for humanizing LM609 that retain integrin $\alpha_v\beta_3$ binding activity, integrin $\alpha_v\beta_3$ binding specificity or integrin $\alpha_v\beta_3$-inhibitory activity, Brooks et al. appears to describe the desirability of humanizing LM609 (column 17, line 62, to column 18, line 3). It is interesting to note that the humanized LM609 antibody alleged to be described and enabled in Brooks et al., 1998, U.S. Patent No. 5,753,230, which was filed March 18, 1994, does not appear to have been made and published until 1998 (Rader et al., *supra*). Applicant contends that Brooks et al., 1998, does not teach humanized LM609 antibody but at most states a problem to be solved. Furthermore, Brooks et al., 1998, does not teach the claimed LM609 CDR-grafted antibody having a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable region amino acid sequence having greater than 79% identity with SEQ ID NO:4.

In regard to the claimed nucleic acids, Applicant respectfully submits that Brooks et al., 1998, does not teach any of the nucleic acids having the structural characteristics of the specifically recited SEQ ID NOS. In particular, Brooks et al., 1998, does not teach nucleic acids encoding a heavy or light chain variable region nucleotide sequence referenced as SEQ ID NOS: 1 or 3. Accordingly, Applicant respectfully submits that Brooks et al., 1998, does not teach the claimed nucleic acids having the structural characteristics of the specifically recited SEQ ID NOS.

Applicant respectfully submits that Brooks et al., 1998, does not teach the claimed LM609 CDR-grafted antibody and polypeptides comprising a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable region amino acid sequence having greater than 79% identity with SEQ ID NO:4. Furthermore, Applicant respectfully submits that Brooks et al., 1998, does not teach a LM609 CDR-grafted antibody and polypeptides comprising a LM609 CDR-grafted heavy chain polypeptide encoded by a LM609 CDR-grafted heavy chain variable region nucleotide sequence referenced as SEQ ID NO:1, or a modification thereof, and a LM609 CDR-grafted light chain polypeptide encoded by a LM609 CDR-grafted light chain variable region nucleotide sequence referenced as SEQ ID NO:3, or a modification thereof, having integrin $\alpha_v\beta_3$ binding activity, integrin $\alpha_v\beta_3$ binding specificity or integrin $\alpha_v\beta_3$-inhibitory activity, as in new claims 26-31. Moreover, Applicant submits that Brooks et al., 1998, does not teach the claimed nucleic acids referenced as SEQ ID NOS:1 and 3. Therefore, Applicant respectfully submits that the claimed antibodies and nucleic

acids, which are referenced as specifically recited SEQ ID NOS, are novel over Brooks et al., 1998. Accordingly, Applicant respectfully requests that this rejection under 35 U.S.C. § 102(e) be withdrawn.

Claims 1-18 stand rejected under 35 U.S.C. § 102(f) because Applicant allegedly did not invent the claimed subject matter. The Office Action alleges that Applicant's arguments, the Huse Declaration filed in the response mailed December 9, 1998, U.S. Patent 5,753,230, issued May 19, 1998 (Brooks et al., 1998), and Biotechnology Newswatch articles, dated January 16, 1995, and February 6, 1995, present an ambiguity with regard to the inventorship of the claimed invention.

Applicant maintains that inventorship has been reviewed and determined to be correct. Dr. Huse has been determined to be the sole inventor of the claimed LM609 CDR-grafted antibody and encoding nucleic acids. In regard to Dr. Cheresh, Applicant maintains that Dr. Cheresh could be considered, at most, a scientific collaborator but not an inventor of the claimed antibodies and nucleic acids referenced as specifically recited SEQ ID NOS.

In regard to Brooks et al., 1998, Applicant respectfully submits that the claimed compositions are novel over Brooks et al., 1998, as described above. Specifically, claims 1, 15 and 17, as amended, are directed to LM609 CDR-grafted antibody and polypeptides comprising a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable

region amino acid sequence having greater than 79% identity with SEQ ID NO:4.

In contrast, Brooks et al., 1998, does not teach the claimed human acceptor framework sequences with LM609 CDRs corresponding to SEQ ID NOS:2 and 4 for LM609 CDR-grafted antibody, as discussed above. Therefore, Applicant respectfully submits that Brooks et al., 1998, does not teach the claimed antibodies having the structural characteristics of the specifically recited SEQ ID NOS.

Furthermore, Applicant respectfully submits that Brooks et al., 1998, does not teach the claimed nucleic acids encoding a heavy or light chain variable region nucleotide sequence referenced as SEQ ID NOS: 1 or 3. As discussed above, Applicant respectfully submits that Brooks et al., 1998, does not teach the claimed nucleic acids having the structural characteristics of the specifically recited SEQ ID NOS.

In light of the above remarks, Applicant respectfully submits that the claimed antibodies and nucleic acids are novel over Brooks et al., 1998. Moreover, as discussed below, Applicant respectfully submits that the claimed antibodies and nucleic acids are unobvious over Brooks et al., 1998. Accordingly, Applicant respectfully requests that the inventorship issues raised with respect to Brooks et al., 1998, be withdrawn.

In regard to the Declaration by Dr. Huse submitted with the previous response mailed December 9, 1998, this previously

filed Declaration was directed to the issue of alleged public use or on sale activity. The statement of conception in this Declaration by Dr. Huse was there to put in perspective the relationship of how certain materials were obtained and maintained under the control of Ixsys.

In summary, Applicant respectfully submits that Dr. Huse is the sole inventor of the claimed LM609 CDR-grafted antibody and encoding nucleic acids having the structural characteristics of the specifically recited SEQ ID NOS and, therefore, that inventorship of the above-identified application is correct. Accordingly, Applicant respectfully requests that the rejection of the claims under 35 U.S.C. § 102(f) be withdrawn.

Claims 1 and 15-18 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Brooks et al., Cell 79:1157-1164 (1994)(Brooks et al., 1994). The Office Action alleges that the specification and Applicant's previously filed arguments indicate that "substantially the same" encompasses LM609 or LM609 grafted antibody and that the breadth of the claims reciting "substantially the same" variable region sequences reads on the LM609 antibody described by Brooks et al., 1994.

Applicant maintains that the claimed compositions directed to a LM609 CDR-grafted antibody are novel over Brooks et al., 1994. In particular, claims 1, 15 and 17, as amended, are directed to LM609 CDR-grafted antibody and polypeptides comprising a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable region amino

acid sequence having greater than 79% identity with SEQ ID NO:4. In contrast to the mouse antibody described in Brooks et al., 1994, Applicant's claimed antibodies have human acceptor framework sequences with LM609 CDRs and, therefore, are non-mouse antibodies (page 8, line 15, to page 9, line 16). Brooks et al., 1994, does not teach the claimed human acceptor framework sequences with LM609 CDRs corresponding to SEQ ID NOS:2 and 4 for LM609 CDR-grafted antibody. Therefore, Brooks et al., 1994, does not teach the claimed non-mouse antibodies and does not teach any of the structural characteristics of the antibodies recited in the claims. Accordingly, Applicant respectfully requests that this rejection under 35 U.S.C. § 102(b) be withdrawn.

Claims 1 and 15-18 also stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Choi et al., J. Vascular Surg. 19:125-134 (1994). The Office Action alleges that the breadth of the claims reciting "substantially the same" variable region sequences reads on the LM609 antibody described by Choi et al.

Applicant maintains that the claimed compositions directed to a LM609 CDR-grafted antibody are novel over Choi et al. In particular, claims 1, 15 and 17, as amended, are directed to LM609 CDR-grafted antibody and polypeptides comprising a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable region amino acid sequence having greater than 79% identity with SEQ ID NO:4. In contrast to the mouse antibody described in Choi et al., Applicant's claimed antibodies have human acceptor framework sequences with LM609 CDRs and, therefore, are non-mouse

antibodies (page 8, line 15, to page 9, line 16). Choi et al. does not teach the claimed human acceptor framework sequences with LM609 CDRs corresponding to SEQ ID NOS:2 and 4 for LM609 CDR-grafted antibody. Therefore, Choi et al. does not teach the claimed non-mouse antibodies and does not teach any of the structural characteristics of the antibodies recited in the claims. Accordingly, Applicant respectfully requests that this rejection under 35 U.S.C. § 102(b) be withdrawn.

Claims 1 and 15-18 stand rejected under 35 U.S.C. 102(a)(e) as allegedly anticipated by Kim et al., U.S. Patent No. 5,578,704, issued November 26, 1996. The Office Action alleges that the breadth of the claims reciting "substantially the same" variable region sequences reads on the LM609 antibody described by Kim et al.

Applicant maintains that the claimed compositions directed to a LM609 CDR-grafted antibody are novel over Kim et al. In particular, claims 1, 15 and 17, as amended, are directed to LM609 CDR-grafted antibody and polypeptides comprising a variable region amino acid sequence having greater than 88% identity with SEQ ID NO:2 and a variable region amino acid sequence having greater than 79% identity with SEQ ID NO:4. In contrast to the mouse antibody described in Kim et al., Applicant's claimed antibodies have human acceptor framework sequences with LM609 CDRs and, therefore, are non-mouse antibodies (page 8, line 15, to page 9, line 16). Kim et al. does not teach the claimed human acceptor framework sequences with LM609 CDRs corresponding to SEQ ID NOS:2 and 4 for LM609 CDR-grafted antibody. Therefore, Kim et al. does not teach the

claimed non-mouse antibodies and does not teach any of the structural characteristics of the antibodies recited in the claims. Accordingly, Applicant respectfully requests that this ground for rejection be withdrawn.

REJECTIONS UNDER 35 U.S.C. § 103

Claims 1-18 stand rejected under 35 U.S.C. § 103 as allegedly unpatentable over Brooks et al., U.S. Patent 5,753,230 (Brooks et al., 1998), Brooks et al., Cell 79:1157-1164 (1994)(Brooks et al., 1994), Choi et al., J. Vascular Surg. 19:125-134 (1994), or Kim et al., U.S. Patent No. 5,578,704, in view of the known art related to gene cloning and expression strategies for deriving recombinant antibodies and fragments thereof. The Office Action states that the newly added reference Brooks et al., 1998, describes the LM609 antibody and humanized forms thereof and claims methods of using LM609 antibody and humanized forms thereof.

Applicant respectfully maintains that the claimed compositions directed to LM609 CDR-grafted antibody and encoding nucleic acids are novel and unobvious over the cited references. The claims recite structural characteristics of the claimed antibodies and encoding nucleic acids, which are not taught or suggested in any of the cited references.

Specifically, the claims directed to a LM609 CDR-grafted antibody recite a heavy chain polypeptide referenced as SEQ ID NO:2 and a light chain polypeptide referenced as SEQ ID

NO:4. Additional claims are directed to nucleic acids encoding heavy or light chain polypeptides comprising a variable region nucleotide sequence, or a modification thereof that does not change the encoded amino acid sequence, shown in Figure 1A (SEQ ID NO:1); or a light chain variable region nucleotide sequence, or a modification thereof that does not change the encoded amino acid sequence, shown in Figure 1B (SEQ ID NO:3). Furthermore, new claims 26-31 are directed to a LM609 CDR-grafted antibody and polypeptides comprising a LM609 CDR-grafted heavy chain polypeptide encoded by a LM609 CDR-grafted heavy chain variable region nucleotide sequence referenced as SEQ ID NO:1, or a modification thereof, and a LM609 CDR-grafted light chain polypeptide encoded by a LM609 CDR-grafted light chain variable region nucleotide sequence referenced as SEQ ID NO:3, or a modification thereof, having integrin $\alpha_v\beta_3$ binding activity, integrin $\alpha_v\beta_3$ binding specificity or integrin $\alpha_v\beta_3$-inhibitory activity.

In regard to claims directed to LM609 CDR-grafted antibody, Applicant respectfully submits that, in contrast to the claimed antibodies, neither Brooks et al., 1998, Brooks et al., 1994, Choi et al., or Kim et al., alone or in combination, teach or suggest any of the structural features specifically recited in the claims. None of the cited references teach or suggest any structural characteristics of an antibody comprising a variable region amino acid sequence referenced as SEQ ID NOS:2 and 4.

In particular, Brooks et al., 1998, does not teach or suggest the claimed human acceptor framework sequences with LM609 CDRs corresponding to SEQ ID NOS:2 and 4 for LM609 CDR-grafted

antibody. Applicants contend, as discussed above, that Brooks et al. does not teach humanized LM609 antibody but at most states a problem to be solved. Therefore, Applicant respectfully submits that Brooks et al., 1998, does not teach or suggest the claimed antibodies having the human acceptor framework sequences with LM609 CDRs and the structural characteristics of the specifically recited SEQ ID NOS.

In regard to the claimed nucleic acid molecules encoding a heavy or light chain variable region nucleotide sequence referenced as SEQ ID NOS:1 or 3, Applicant respectfully submits that, in contrast to the claimed nucleic acids, Brooks et al., 1998, does not teach or suggest any of the structural features specifically recited in the claims as SEQ ID NOS. As discussed in the previous responses mailed February 4, 1998, and December 9, 1998, "the existence of a general method of isolating cDNA or DNA molecules is essentially irrelevant to the question whether the specific molecules themselves would have been obvious, in the absence of other prior art that suggest the claimed DNAs." *In re Deuel*, 34 USPQ 2d 1215 (Fed. Cir. 1995). Since Brooks et al., 1998, does not teach or suggest the nucleic acids having the structural characteristics of the specifically recited SEQ ID NOS, Applicant respectfully submits that Brooks et al., 1998, does not render obvious the claimed nucleic acids.

In regard to Brooks et al., 1994, Choi et al. and Kim et al., Applicant respectfully submits that these cited references describe the mouse antibody, not Applicant's claimed non-mouse antibodies having human acceptor framework sequences

with LM609 CDRs. Similarly, Brooks et al., 1994, Choi et al., and Kim et al. do not teach or suggest the claimed nucleic acids encoding the non-mouse antibodies having human acceptor framework sequences with LM609 CDRs. Therefore, Applicant respectfully submits that Brooks et al., 1994, Choi et al. and Kim et al., alone or in combination with Brooks et al., 1998, do not render obvious the claims directed to antibodies or nucleic acids that recite specific SEQ ID NOS.

In regard to newly added claims 26-31, which are directed to a LM609 CDR-grafted antibody and polypeptides comprising a LM609 CDR-grafted heavy chain polypeptide encoded by a LM609 CDR-grafted heavy chain variable region nucleotide sequence referenced as SEQ ID NO:1, or a modification thereof, and a LM609 CDR-grafted light chain polypeptide encoded by a LM609 CDR-grafted light chain variable region nucleotide sequence referenced as SEQ ID NO:3, or a modification thereof, having integrin $\alpha_v\beta_3$ binding activity, integrin $\alpha_v\beta_3$ binding specificity or integrin $\alpha_v\beta_3$-inhibitory activity. Applicant respectfully submits that Brooks et al., 1998, Brooks et al., 1994, Choi et al. and Kim et al., alone or in combination, do not teach or suggest a LM609 CDR-grafted antibody or polypeptide encoded by nucleotide sequences specifically recited as SEQ ID NOS:1 and 3. Accordingly, Applicant respectfully submits that these cited references, alone or in combination, do not render obvious new claims 26-31.

In summary, Applicant respectfully submits that the claimed antibodies and nucleic acids having the structural features specifically recited in the claims as SEQ ID NOS are

novel and unobvious over any of Brooks et al., 1998, Brooks et al., 1994, Choi et al., or Kim et al., alone or in combination. Therefore, the rejection of these claims under 35 U.S.C. § 103 as allegedly obvious is respectfully requested to be withdrawn.

CONCLUSION

In light of the amendments and remarks herein, Applicant submits that the claims are now in condition for allowance and respectfully request a notice to this effect. The Examiner is invited to call Cathryn Campbell or the undersigned agent if there are any questions.

Respectfully submitted,

September 20, 1999
Date

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Serial No.: 08/790,540
Filed: January 30, 1997

EXHIBIT 1

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BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

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DOC DATE: 03/31/1997

ASSIGNOR:
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DOC DATE: 03/17/1997

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MAIL ROOM 30 APR 16 1997 PAT. & TRADEMARK OFFICE

OIPE JC67 SEP 23 1999 PATENT & TRADEMARK OFFICE

To the Honorable Commissioner of Patents and Trademarks: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):
William D. Huse
Scott M. Glaser

Additional names(s) of conveying party(ies) attached? ☐ Yes ☒ No

2. Name and address of receiving party(ies):

Name: **IXSYS, INCORPORATED**

Internal Address: _____

Street Address: **3550 Dunhill Road**

City: **San Diego** State: **CA** ZIP: **92121**

Additional name(s) & address(es) attached? ☐ Yes ☒ No

3. Nature of conveyance:

☒ Assignment ☐ Merger
☐ Security Agreement ☐ Change of Name
☐ Other _____

Execution Date: **03/31/97; 03/17/97**

4. Application number(s) or registration numbers(s):

If this document is being filed together with a new application, the execution date of the application is: _____

A. Patent Application No.(s)

08/791,391

B. Patent No.(s)

05-08-1997

100412265

Additional numbers attached? ☐ Yes ☒ No

5. Name and address of party to whom correspondence concerning document should be mailed:

Name: **Cathryn Campbell**

Internal Address: **CAMPBELL & FLORES LLP**

Street Address: **4370 La Jolla Village Drive, Suite 700**

City: **San Diego** State: **CA** ZIP: **92122**

6. Total number of applications and patents involved: **1**

7. Total fee (37 CFR 3.41):.....$ **40.00**

☒ Enclosed

☐ Authorized to be charged to deposit account

8. Deposit account number:

03-0370

(Attach duplicate copy of this page if paying by deposit account)

DO NOT USE THIS SPACE

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

David A. Gay, Registration No. 39,200 — Name of Person Signing

Signature

April 14, 1997 — Date

Total number of pages including cover sheet, attachments, and document: **5**

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7-14-97

JOINT

ASSIGNMENT

This Assignment is made by William D. Huse of Del Mar, California, and Scott M. Glaser of Seattle, Washington, Assignors, to IXSYS, INCORPORATED, Assignee, having a place of business at 3550 Dunhill Road, San Diego, California 92121.

WHEREAS, Assignors have invented a new and useful invention entitled ANTI-$\alpha_v\beta_3$ RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE for which an application for United States Letters Patent was filed on January 30, 1997, in the United States Patent and Trademark Office, bearing Serial No.08/791,391 and identified as Attorney Docket No. P-IX 1482;

WHEREAS, Assignors believe themselves to be the original inventors of the invention disclosed and claimed in said application for Letters Patent; and

WHEREAS, the parties desire to have a recordable instrument assigning the entire right, title and interest in and to said invention, said application and any Letters Patent that may be granted for said invention in the United States and throughout the world;

NOW, THEREFORE, in accordance with the obligations to assign the invention and other good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged,

Assignors sell, assign, and transfer to Assignee, the entire right, title, and interest in and to said invention, said application and any Letters Patent that may be granted for said invention in the United States and throughout the world, including the right to file foreign applications directly in the name of the Assignee and to claim for any such foreign applications any priority rights to which such applications are entitled under international conventions, treaties, or otherwise.

Assignors agree that, upon request and without further compensation, but at no expense to Assignors, they and their legal representatives and assigns will do all lawful acts, including the execution of papers and the giving of testimony,. that may be necessary or desirable for obtaining, sustaining, reissuing, or enforcing Letters Patent in the United States and throughout the world for said invention, and for perfecting, recording, or maintaining the title of Assignee, its successors and assigns, to said invention, said application, and any Letters Patent granted for said invention in the United States and throughout the world.

Assignors represent and warrant that they have not granted and will not grant to others any rights inconsistent with the rights granted herein.

Assignors authorize and request the Commissioner of Patents and Trademarks of the United States and of all foreign countries to issue any Letters Patent granted for said invention, whether on said application or on any subsequently filed division, continuation, continuation-in-part or reissue application, to Assignee, its successors and assigns, as the assignee of the entire interest in said invention.

IN WITNESS WHEREOF, Assignors have executed this Assignment on the date(s) provided below.

Assignor: William D. Huse

Signature

STATE OF California)
COUNTY OF San Diego)

On March 31, 1997, before me, Donna G. Frick, a Notary Public, personally appeared William D. Huse, X personally known to me - OR - ___ proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

WITNESS by hand and official seal.

Donna G. Frick
(Signature of Notary)

DONNA G FRICK
COMM. #1126339
NOTARY PUBLIC-CALIFORNIA
SAN DIEGO COUNTY
My Comm. Exp. Feb. 6, 2001

Assignor: Scott M. Glaser

Scott M Glaser

Signature

STATE OF Washington)
COUNTY OF King)

On March 17 1997, before me, Jan S. Hedley, a Notary Public, personally appeared Scott M. Glaser, ✓ personally known to me - OR - ___ proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

WITNESS by hand and official seal.

Jan S Hedley

(Signature of Notary)

Inventors: William D. Huse
Serial No.: 08/790,540
Filed: January 30, 1997

EXHIBIT 2

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

AUGUST 15, 1997

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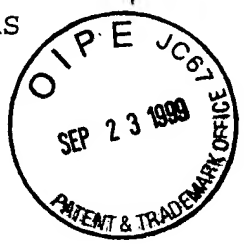


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CAMPBELL & FLORES, LLP
DAVID A. GAY
4370 LA JOLLA VILLAGE DRIVE
SUITE 700
SAN DIEGO, CA 92122

UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE ASSIGNMENT SEARCH ROOM ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

PLEASE REVIEW ALL INFORMATION CONTAINED ON THIS NOTICE. THE INFORMATION CONTAINED ON THIS RECORDATION NOTICE REFLECTS THE DATA PRESENT IN THE PATENT AND TRADEMARK ASSIGNMENT SYSTEM. IF YOU SHOULD FIND ANY ERRORS OR HAVE QUESTIONS CONCERNING THIS NOTICE, YOU MAY CONTACT THE EMPLOYEE WHOSE NAME APPEARS ON THIS NOTICE AT 703-308-9723. PLEASE SEND REQUEST FOR CORRECTION TO: U.S. PATENT AND TRADEMARK OFFICE, ASSIGNMENT DIVISION, BOX ASSIGNMENTS, NORTH TOWER BUILDING, SUITE 10C35, WASHINGTON, D.C. 20231.

RECORDATION DATE: 06/02/1997

REEL/FRAME: 8608/0497
NUMBER OF PAGES: 4

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

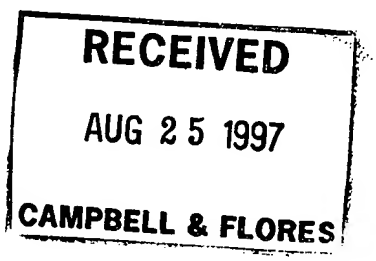
ASSIGNOR:
HUSE, WILLIAM D.

DOC DATE: 03/31/1997

ASSIGNEE:
IXSYS, INCORPORATED
3550 DUNHILL ROAD
SAN DIEGO, CALIFORNIA 92121

SERIAL NUMBER: 08790540
PATENT NUMBER:

FILING DATE: 01/30/1997
ISSUE DATE:



MAURICE CARTER, EXAMINER
ASSIGNMENT DIVISION
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SHEET 1 OF 2

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

To the Honorable Commissioner o[f Patents and Trademarks]: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):
William D. Huse

Additional names(s) of conveying party(ies) attached? ☐ Yes ☒ No

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Name: **IXSYS, INCORPORATED**

Internal Address:

Street Address: **3550 Dunhill Road**

City: **San Diego** State: **CA** ZIP: **92121**

Additional name(s) & address(es) attached? ☐ Yes ☒ No

3. Nature of conveyance:

☒ Assignment ☐ Merger

☐ Security Agreement ☐ Change of Name

☐ Other

Execution Date: **March 31, 1997**

4. Application number(s) or registration numbers(s):

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s)

08/790,540

B. Patent No.(s)

Additional numbers attached? ☐ Yes ☒ No

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Street Address: **4370 La Jolla Village Drive, Suite 700**

City: **San Diego** State: **CA** ZIP: **92122**

6. Total number of applications and patents involved: **1**

7. Total fee (37 CFR 3.41):.....$ **40.00**

☒ Enclosed

☐ Authorized to be charged to deposit account

8. Deposit account number:

03-0370

(Attach duplicate copy of this page if paying by deposit account)

DO NOT USE THIS SPACE

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

David A. Gay, Registration No. 39,200 — Name of Person Signing

Signature

May 28, 1997 — Date

Total number of pages including cover sheet, attachments, and document: **4**

6/25/1997 SBELTT 00000040 08790540
1 FC:581 40.00 OP

MRU 6-24-97

SOLE

ASSIGNMENT

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WHEREAS, Assignor has invented a new and useful invention entitled ANTI-$\alpha_v\beta_3$ RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE for which an application for United States Letters Patent was filed on January 30, 1997 in the United States Patent and Trademark Office, bearing Serial No.08/790,540 and identified as Attorney Docket No. P-IX 2405.

WHEREAS, Assignor believes himself to be the original inventor of the invention disclosed and claimed in said application for Letters Patent; and

WHEREAS, the parties desire to have a recordable instrument assigning the entire right, title and interest in and to said invention, said application and any Letters Patent that may be granted for said invention in the United States and throughout the world;

NOW, THEREFORE, in accordance with the obligations to assign the invention and other good and valuable consideration, the receipt and sufficiency of which are hereby acknowledged, Assignor sells, assigns, and transfers to Assignee, the entire right, title, and interest in and to said invention, said application and any Letters Patent that may be granted for said invention in the United States and throughout the world, including the right to file foreign applications directly in the name of the Assignee and to claim for any such foreign applications any priority rights to which such applications are entitled under international conventions, treaties, or otherwise.

Assignor agrees that, upon request and without further compensation, but at no expense to Assignor, he and his legal representatives and assigns will do all lawful acts, including the execution of papers and the giving of testimony, that may be necessary or desirable for obtaining, sustaining, reissuing, or enforcing Letters Patent in the United States and throughout the world for said invention, and for perfecting, recording, or maintaining the title of Assignee, its successors and assigns, to said invention, said application, and any Letters Patent granted for said invention in the United States and throughout the world.

Assignor represents and warrants that he has not granted and will not grant to others any rights inconsistent with the rights granted herein.

Assignor authorizes and requests the Commissioner of Patents and Trademarks of the United States and of all foreign countries to issue any Letters Patent granted for said invention, whether on said application or on any subsequently filed divisional, continuation, continuation-in-part or reissue application, to Assignee, its successors and assigns, as the assignee of the entire interest in said invention.

IN WITNESS WHEREOF, Assignor has executed this Assignment on the date(s) provided below.

Assignor: William D. Huse

Signature

STATE OF California)
COUNTY OF San Diego)

On March 31, 1997, before me, Donna G. Frick, a notary public, personally appeared William D. Huse, X personally known to me - OR - ___ proved to me on the basis of satisfactory evidence to be the person(s) whose name(s) is/are subscribed to the within instrument and acknowledged to me that he/she/they executed the same in his/her/their authorized capacity(ies), and that by his/her/their signature(s) on the instrument the person(s), or the entity upon behalf of which the person(s) acted, executed the instrument.

WITNESS by hand and official seal.

Donna G. Frick
(Signature of Notary)

DONNA G. FRICK
COMM. #1126339
NOTARY PUBLIC-CALIFORNIA
SAN DIEGO COUNTY
My Comm. Exp. Feb. 6, 2001

Inventors: William D. Huse
Serial No.: 08/790,540
Filed: January 30, 1997

EXHIBIT 3

PATENT
Our Docket: P-IX 2405

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: William D. Huse	)	Group Art Unit: 1644
Serial No.: 08/790,540	)	Examiner: P. Gambel
Filed: 01/30/97	)	
For: ANTI-$\alpha_v\beta_3$ RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE	)	

#19

Asst. Commissioner for Patents
Washington, D.C. 20231

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Sir:

I, William D. Huse, declare as follows:

1) I am the William D. Huse who is named as an inventor on the above-identified patent application.

2) I have reviewed the Office Action mailed March 18, 1999, and understand that the claims of the subject application stand rejected, in part, because the claimed LM609 CDR-grafted antibody, which is an antibody comprising a variable region amino acid sequence referenced as SEQ ID NOS:2 and 4, is alleged to have been on sale or in public use more than one year prior to the filing of the above-identified application.

3) The LM609 hybridoma was brought to Ixsys, Inc., and the LM609 heavy and light chain variable region cDNA was cloned. LM609 grafted antibodies, including Vitaxin, were generated and developed having $\alpha_v\beta_3$ inhibitory activity.

4) Agreements made with third parties were confidential, and Ixsys maintained control of all antibody materials, including any Vitaxin or LM609 grafted antibody, any nucleic acid encoding Vitaxin or LM609 grafted antibody, any cell lines containing nucleic acid encoding Vitaxin or LM609 grafted antibody, and any related materials or information. As evidence that Ixsys maintained control and confidentiality of these materials in agreements with third parties, attached herewith as Exhibit A are portions of an agreement with Celltech Therapeutics Limited and its affiliate, Celltech Biologics PLC. In the agreement on page 33, section 1.1.12, the "product" referred to in the agreement means the CDR grafted antibody. As indicated on page 34, section 2.3 of the agreement, Celltech was obligated to maintain safe keeping of the materials and products, including any Vitaxin or LM609 grafted antibody, nucleic acids encoding any Vitaxin or LM609 grafted antibody, cell lines containing nucleic acids encoding any Vitaxin or LM609 grafted antibody, and information related to any Vitaxin or LM609 grafted antibody and encoding nucleic acids, and to assure that any parties involved in testing of the product are subject to the same obligations of confidence as Celltech.

5) Ixsys maintained control and confidentiality of Vitaxin and LM609 grafted antibody, nucleic acids encoding Vitaxin and LM609 grafted antibody, cell lines containing nucleic acids encoding Vitaxin and LM609 grafted antibody, related materials and their use during any involvement with third parties. Neither Vitaxin nor LM609 grafted antibody were on sale or in public use more than one year prior to January 30, 1997.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

9/20/99
Date

William D. Huse

1.1.19 "Valid and Enforceable Claim" means a claim in an issued, unexpired patent which has not been held invalid or unenforceable in an unappealed decision of a court or other competent body having jurisdiction thereof.

1.2 Unless the context requires otherwise, words and phrases defined in any other part of the Agreement shall bear the same meanings as in these Standard Terms, references to the singular number include the plural and vice versa, references to Schedules are references to schedules to the Agreement, and references to Clauses are references to clauses of these Standard Terms.

2. Supply of the Customer Materials

2.1 Prior to or immediately following the date of the Agreement the Customer shall supply to Celltech the information referred to in Clause 1 (i) of Schedule 2 hereto followed at the appropriate time by supply of the Customer Materials and Customer Information, together with full details of any hazards relating to the Customer Materials, their storage and use. Property in the Customer Materials supplied to Celltech shall remain vested in the Customer.

2.2 The Customer hereby grants Celltech the non-exclusive right to use the Customer Materials and the Customer Information for the purpose of the Agreement. Celltech hereby undertakes not to use the Customer Materials or the Cell Line containing the Customer Materials or the Customer Information (or any part thereof) for any other purpose.

2.3 Celltech shall :

2.3.1 be responsible for the safe keeping of the Customer Materials in its possession and shall at all times keep the Customer Materials secure and safe from loss and damage in such manner as Celltech shall in its sole discretion determine;

2.3.2 not part with possession of the Customer Materials or the Product, save for the purpose of tests at the Testing Laboratories.

2.3.3 procure that all Testing Laboratories are subject to obligations of confidence substantially in the form of those obligations of confidence imposed on Celltech under these Standard Terms and where relevant procure that such Testing Laboratories are subject to obligations to comply with GLP.

2.4 Celltech shall not be liable for any loss, damage, costs or expenses of any nature, whether direct or consequential, occasioned by the carrying out (in whole or in part) of tests or the failure to carry out tests by Testing Laboratories, provided that such liability is not a direct result of the negligence or wilful misconduct of Celltech or Celltech's employees and that Celltech shall inform Customer of the occurrence of such loss, damage, costs or expenses as soon as is reasonably possible .

2.5 The Customer warrants to Celltech that to the best of its knowledge and belief :

2.5.1 the Customer is and shall at all times throughout the duration of the Agreement remain entitled to supply the Customer Materials and Customer Information to Celltech; and

2.5.2 use by Celltech of the Customer Materials and the Customer Information for the purposes of the Services will not infringe any rights (including, without limitation, any intellectual or industrial property rights) vested in any third party save that subject to the above warranty Customer gives no warranty as to Celltech's entitlement to use gene expression and production technology associated with the Process.

2.6 The Customer undertakes to indemnify and to maintain Celltech promptly indemnified against any loss, damage, costs and expenses of any nature (including court costs and legal fees on a full indemnity basis), whether direct or consequential, and whether or not foreseeable or in the contemplation of Celltech or the Customer, that Celltech may suffer arising out of or incidental to any breach of the warranties given by the Customer under Clause 2.5 above.

2.7 The obligations of the Customer under this Clause 2 shall survive the termination for whatever reason of the Agreement in respect of matters arising during the term of or pursuant to this Agreement.

3. Provision of the Services

3.1 Celltech shall and where relevant shall procure that Celltech Biologics plc shall diligently carry out the Services as provided in Schedule 2 and shall keep Customer informed of the progress of the Services and shall use reasonable endeavours to achieve the estimated timescales set out therein.

3.2 Notwithstanding the provisions of Clause 3.1 the timescales set down for the performance of the Services (including without limitation the dates for production and delivery of Product) and the quantities of Product for delivery set out in Schedule 2 are estimated only. Time of performance of the Services, time of production and time of delivery shall not be of the essence of the Agreement.

3.3 The Customer shall not be entitled to cancel any unfulfilled part of the Services or to refuse to accept the Services on grounds of late performance, late delivery or failure to produce the estimated quantities of Product for delivery. Celltech shall not be liable for any loss, damage, costs or expenses of any nature, whether direct or consequential, occasioned by :

3.3.1 any delay in performance or delivery howsoever caused;

3.3.2 any failure to produce the estimated quantities of Product for delivery.

3.4 Celltech shall comply with all statutory, regulatory and similar legislative requirements from time to time applicable to the Services under the laws of England. If the Customer requests Celltech to comply with any foreign statutory, regulatory or similar legislative requirements Celltech shall use reasonable commercial endeavours to do so provided that :

3.4.1 the Customer shall be responsible for informing Celltech in writing of the precise foreign requirements which the Customer is requesting Celltech to observe; and

Inventors: William D. Huse
Serial No.: 08/790,540
Filed: January 30, 1997

EXHIBIT 4

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 8910–8915, July 1998
Medical Sciences

A phage display approach for rapid antibody humanization: Designed combinatorial V gene libraries

CHRISTOPH RADER*, DAVID A. CHERESH†, AND CARLOS F. BARBAS III*‡

*Skaggs Institute for Chemical Biology and Department of Molecular Biology and †Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, CA 92037

Communicated by Frank J. Dixon, The Scripps Research Institute, La Jolla, CA, May 21, 1998 (received for review March 18, 1998)

ABSTRACT **The development of a new strategy for antibody humanization is described. This strategy incorporates key recognition sequences from the parental rodent antibody into a phage display-based selection strategy. The original sequences of the third complementarity-determining regions (CDRs) of heavy and light chains, HCDR3 and LCDR3, were maintained and all other sequences were replaced by human sequences selected from phage-displayed antibody libraries. This approach was applied to the humanization of mouse mAb LM609 that is directed to human integrin $\alpha_v\beta_3$ and has potential applicability in cancer therapy as an antiangiogenic agent. We demonstrate this approach (*i*) provides a rapid route for antibody humanization constraining the content of original mouse sequences in the final antibodies to the most hypervariable of the CDRs; (*ii*) generates several humanized versions with different sequences at the same time; (*iii*) results in affinities as high as or higher than the affinity of the original antibody; and (*iv*) retains the antigen and epitope specificity of the original antibody. The production of multiple humanized variants may present advantages in the selection of antibodies that are more readily expressed on a large scale and could be important in therapeutic regimens that call for long-term treatment with antibodies in which antiidiotypic responses might be avoided by administration of alternative antibodies.**

Since the development of the hybridoma approach (1), a large number of rodent mAbs with specificity for antigens of therapeutic interest have been generated and characterized. The fact that rodent antibodies are highly immunogenic in humans, however, severely limits their clinical applications, especially when repeated administration is required for therapy. As a means of circumventing this limitation, several strategies have been developed to convert rodent antibody sequences into human antibody sequences, a process termed antibody humanization. Ideally, antibody humanization must not diminish specificity and affinity toward the antigen whereas immunogenicity must be completely eliminated. It has become apparent that the accomplishment of both aims is usually a time-consuming and costly undertaking with even the most current humanization strategies. Here, we report the development of a new humanization strategy that combines rational design with combinatorial selections using phage display. We demonstrate that this approach provides a rapid route to antibody humanization and demonstrate its application to the humanization of mouse mAb LM609 which is directed against the human integrin $\alpha_v\beta_3$. We chose LM609 as a model antibody for our humanization strategy because of its clinical potential. Recent findings by Brooks *et al.* (2–4) in a chorioallantoic membrane model and a severe combined immunodeficient mouse/human skin chimeric model have shown that LM609, when administered i.v., is able to reduce growth and metastasis of human tumors due to the inhibition of angiogenesis induced by the tumors. These findings suggest that integrin $\alpha_v\beta_3$ may be a target and LM609 a tool for cancer therapy.

MATERIALS AND METHODS

Proteins and Cell Lines. Human integrin $\alpha_v\beta_3$ was purified from human placenta as described (5). Human integrin $\alpha_{IIb}\beta_3$ was purchased from Enzyme Research Laboratories (South Bend, IN). mAb LM609 was described previously (6) and mAb AP3 was kindly provided by P. Newman (Milwaukee Blood Center, Milwaukee, WI). LM609 Fab was generated from IgG by digestion with immobilized papain using the ImmunoPure Fab Preparation kit from Pierce and separated from Fc and undigested IgG by three consecutive runs on a protein A column. CS-1 hamster cells were transfected with either human β_3 or β_5 cDNA as described (7) and maintained in RPMI 1640 supplemented with 10% fetal calf serum and 500 μg/ml G-418 (Life Technologies, Gaithersburg, MD) at 37°C and in 7% CO_2.

cDNA Cloning of LM609. Total RNA was prepared from 10^8 LM609 hybridoma cells (6) using the RNA Isolation kit from Stratagene. Reverse transcription and PCR amplification of the Fd fragment- and light chain-coding sequences were performed essentially as described (8). Fd fragment- and light chain-coding PCR products were cut with *Xho*I/*Spe*I and *Sac*I/*Xba*I, respectively, and ligated sequentially into the appropriately digested phagemid vector pComb3H (9). The ligation products were introduced into *Escherichia coli* strain XL1-Blue by electrotransformation and subsequent steps were as described (10) to produce phage displaying Fab on their surface. Phage were selected by panning (10) against immobilized human integrin $\alpha_v\beta_3$. After two panning rounds, single clones were analyzed for LM609 Fab expression. Supernatants from cultures that had been induced by the addition of isopropyl β-D-thiogalactopyransoside (10) were tested for binding to $\alpha_v\beta_3$ by ELISA using goat anti-mouse $F(ab')_2$ conjugated to alkaline phosphatase (Pierce) as secondary antibody. The sequence of Fd fragment- and light chain-coding sequences of positive clones was determined by DNA sequencing.

Amplification of Human Light Chain and Fd Fragment Sequences. Total RNA was prepared from bone marrow of five healthy donors supplied by Poietic Technologies (Germantown, MD) shortly after aspiration using TRI REAGENT (Molecular Research Center, Cincinnati, OH) and was further purified by lithium chloride precipitation (11). First-strand cDNA was synthesized using the SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis kit with oligo(dT) priming (Life Technologies). The generated five first-strand cDNAs were subjected to separate PCR amplifications. V_κ, V_λ, and V_H sequences of each of the first-strand cDNAs were amplified using the primers listed below. All amplifications were performed under standard PCR conditions using *Taq* polymerase (Pharmacia).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CDR, complementarity-determining region; FR, framework region.

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While the sense primers hybridize to sequences that encode the N-terminal amino acids of the various V_κ, V_λ, and V_H families, the antisense primers hybridize to sequences that encode the C-terminal amino acids of framework region 3 (FR3) of V_κ, V_λ, or V_H, respectively, which are highly conserved (12). The primers used for the amplification of human antibody sequences are V_κ sense primers: HSCK1-F, 5′-GGGCCCAGGCGGCCGAGCTCCAGATGACCCAGTCTCC-3′; HSCK24-F, 5′-GGGCCCAGGCGGCCGAGCTCGTGATGACYCAGTCTCC-3′; HSCK3-F, 5′-GGGCCCAGGCGGCCGAGCTCGTGWTGACRCAGTCTCC-3′; and HSCK5-F, 5′-GGGCCCAGGCGGCCGAGCTCACACTCACGCAGTCTCC-3′; V_κ antisense primers: BKFR3UN, 5′-CAGTAATACACTGCAAAATCTTC-3′; BK2FR3UN and 5′-CAGTAATAAACCCCAACATCCTC-3′; V_λ sense primers: HSCLam1a, 5′-GGGCCCAGGCGGCCGAGCTCGTGBTGACGCAGCCGCCCTC-3′; HSCLam1b, 5′-GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCACCCTC-3′; HSCLam2, 5′-GGGCCCAGGCGGCCGAGCTCGCCCTGACTCAGCCTCCCTCCGT-3′; HSCLam3, 5′-GGGCCCAGGCGGCCGAGCTCGAGCTGACTCAGCCACCCTCAGTGTC-3′; HSCLam4, 5′-GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAATCGCCCTC-3′; HSCLam6, 5′-GGGCCCAGGCGGCCGAGCTCATGCTGACTCAGCCCCACTC-3′; HSCLam70, 5′-GGGCCCAGGCGGCCGAGCTCGGGCAGACTCAGCAGCTCTC-3′; HSCLam78, 5′-GGGCCCAGGCGGCCGAGCTCGTGGTGACYCAGGAGCCMTC-3′; and HSCLam9, 5′-GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCACCTTC-3′; V_λ antisense primer: BLFR3UN, 5′-GCAGTAATAATCAGCCTCRTC-3′; V_H sense primers: HFVH1-F, 5′-GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG-3′; HFVH2-F, 5′-GCTGCCCAACCAGCCATGGCCCAGATCACCTTGAAGGAGTCTGG-3′; HFVH35-F, 5′-GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGGTGSAGTCTGG-3′; and HFVH4-F, 5′-GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG-3′; V_H antisense primer: BFR3UN, 5′-CGCACAGTAATACACGGCCGTGTC-3′.

Construction of a Chimeric Mouse/Human Fd Fragment by Fusing V_H of LM609 to Human C_H1. The phagemid vector pComb3H containing the LM609 Fab sequence was used as a template for amplification of the sequence encoding the N-terminal FR1 through FR3 fragment of the LM609 V_H by the PCR primer pair PELSEQ (5′-ACCTATTGCCTACGGCAGCCG-3′)/BFR3UN (5′-CGCACAGTAATACACGGCCGTGTC-3′). By overlap-extension PCR (13), the PELSEQ/BFR3UN product was fused to a PCR fragment encoding the HCDR3 of LM609, FR4 of V_H, and the entire C_H1 domain of the human anti-gp120 antibody b8 (14). This fragment was generated from the PCR primer pair CR501 (5′-GACACGGCCGTGTATTACTGTGCGCGTCATAACTACGGCAGTTTTGCTTACTGGGGCCAGGGAACCCTG-3′)/CR301 (5′-GAGGAGGAGGAGGAGACTAGTTTTGTCACAAGATTTGGGCTC-3′). FR4 of b8 was chosen because it is identical to FR4 of the LM609 V_H, with the exception of the C-terminal amino acid, which is A for LM609 and S for b8. The product of the overlap-extension PCR was cut with *XhoI*/*SpeI*, ligated into the appropriately digested phagemid vector pComb3H, cloned, and the correct sequence was confirmed by DNA sequencing.

Substitution of the LM609 Light Chain by a Human Light Chain That Contains the LCDR3 of LM609. Using overlap-extension PCR, the amplified human sequences encoding the N-terminal FR1 through FR3 fragment of V_κ and V_λ were fused to PCR fragments encoding the LCDR3 of LM609 coupled to FR4 of human V_κ or V_λ and the human C_κ or C_λ domain. Two κ fragments were generated by the PCR primer pairs CR503 (5′-GAAGATTTTGCAGTGTATTACTGCCAACAGAGTAACAGCTGGCCTCACACGTTTGGCCAGGGGACCAAGCTG-3′)/T7B (5′-AATACGACTCACTATAGGGCG-3′) and CR508 (5′-GAGGATGTTGGGGTTTATTACTGCCAACAGAGTAACAGCTGGCCTCACACGTTTGGCCAGGGGACCAAGCTG-3′)/T7B using the sequence of the anti-gp120 antibody b11 in pComb3 as a template (14). FR4 of b11 was chosen because it is identical to FR4 of the LM609 V_κ, with the exception of the third amino acid, which is G in LM609 and Q in b11. The 23-bp overlap of CR503 with BKFR3UN and CR508 with BK2FR3UN allowed the fusion of the corresponding PCR products by overlap-extension PCR. A λ fragment was generated by the PCR primer pair CR510 (5′-GAYGAGGCTGATTATTACTGCCAACAGAGTAACAGCTGGCCTCACACGTTCGGCGGAGGGACCAAGCTG-3′)/CLext (5′-AGAGAGAGAGAGAGAGAGAGCGCCGTCTAGAATTATGAACATTCTGTAGG-3′) using CLext-primed, first-strand cDNA from human bone marrow as a template. The 21-bp overlap of CR510 with BLFR3UN allowed the fusion of the corresponding PCR products by overlap-extension PCR. The generated light chain-coding sequences were cut with *SacI*/*XbaI* and ligated into the appropriately digested phagemid vector pComb3H that contained the chimeric mouse/human Fd fragment. Electrotransformation of the ligation products into *E. coli* strain ER 2537 (New England Biolabs) resulted in a light chain library consisting of 1.5×10^8 independent transformants. DNA sequencing revealed the correct assembly of the fused fragments. Four rounds of panning against immobilized human integrin $\alpha_V\beta_3$ were carried out using 200 ng of protein in 25 μl of metal buffer [25 mM Tris·HCl (pH 7.5), 137 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$] for coating, 0.05% Tween 20 in Tris-buffered saline for washing, and 10 mg/ml trypsin (Difco) in Tris-buffered saline for elution. Trypsinization was for 30 min at 37°C. The washing steps were increased from 5 in the first round to 10 in the second round and 15 in the third and fourth rounds. The output phage pool of each round was monitored by phage ELISA using sheep anti-M13 conjugated to horseradish peroxidase (Pharmacia) as secondary antibody. After the fourth round, phage were produced from single clones and tested for binding to $\alpha_V\beta_3$ by phage ELISA. Light chain-coding sequences of positive clones were analyzed by DNA sequencing using the primer OMPSEQ (5′-AAGACAGCTATCGCGATTGCAG-3′).

Substitution of the LM609 Fd Fragment by a Human Fd Fragment That Contains the HCDR3 of LM609. Three PCR fragments were fused in one step by overlap-extension PCR. Using the selected phagemids from the light chain panning as a template, fragment 1 was amplified with the PCR primer pair RSC-F (5′-GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC-3′)/lead-B (5′-GGCCATGGCTGGTTGGGCAGC-3′). While the sense primer RSC-F hybridizes to a sequence upstream of the light chain-coding sequence, the antisense primer lead-B hybridizes to a sequence upstream of the Fd fragment-coding sequence. The amplified human sequences encoding FR1 through FR3 of the V_H fragment (see above) were used as fragment 2. Fragment 3 was amplified with the PCR primer pair CR501/HIgG1-B (5′-GCAGAGCCCAAATCTTGTGACACTAGTGGCCAGGCCGGCCAG-3′) using the hybrid mouse/human Fd fragment (see above) as a template. The antisense primer HIgG1-B hybridizes to the 3′ end of the C_H1-coding sequence. Using the 21-bp overlap of lead-B with the HFVH-F primers and the 24-bp overlap of BFR3UN with CR501, the three fragments were fused and amplified with the PCR primer pair RSC-F/RSC-B (5′-GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG-3′). The antisense primer RSC-B overlaps with HIgG1-B. RSC-F and RSC-B introduce two asymmetric *SfiI* sites. To maintain high complexity, separate PCRs were performed for each selected phagemid from the light chain panning (fragment 1) and for each of the five V_H fragment pools derived from the five first-strand cDNA sources (fragment 2). The generated fragments encoding the selected human light chains linked to human Fd fragments

were cut with *Sfi*I and ligated into the appropriately digested phagemid vector pComb3H, generating a library of 3×10^7 independent transformants. DNA sequencing revealed the correct assembly of the fused DNA fragments. Four rounds of panning against immobilized human integrin $\alpha_V\beta_3$ were carried out as described for the light chain panning. The output phage pool of each round was monitored by phage ELISA. After the fourth round, soluble Fab was produced from single clones as described (10) and tested for binding to immobilized $\alpha_V\beta_3$ by ELISA using goat anti-human $F(ab')_2$ conjugated to alkaline phosphatase (Pierce) as secondary antibody. Light chain- and Fd fragment-coding sequences of positive clones were analyzed by DNA sequencing using the primers OMPSEQ and PELSEQ, respectively.

Flow Cytometry. Flow cytometry was performed using a FACScan instrument from Becton Dickinson. For each determination, 5×10^3 untransfected hamster CS-1 cells or hamster CS-1 cells transfected with either human β_3 or β_5 cDNA were analyzed. Indirect immunofluorescence staining was performed with 2 μg/ml Fab in 1% BSA, 25 mM Hepes, and 0.05% sodium azide in PBS supplemented with 1% nonimmune goat serum. A 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-human $F(ab')_2$ (Jackson ImmunoResearch Laboratories) was used for detection. Incubation with primary antibodies was for 1 h, with secondary antibodies for 30 min, at room temperature. Competition experiments were performed by adding a fourfold molar excess of LM609 or AP3 IgG to the incubation mixture with the primary antibodies.

Surface Plasmon Resonance. Association (k_{on}) and dissociation (k_{off}) rate constants for binding of mouse and humanized LM609 Fab to human integrin $\alpha_V\beta_3$ were determined by surface plasmon resonance on a Biacore instrument (Pharmacia). The sensor chip was activated for immobilization with *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-diethyl aminopropyl)carbodiimide according to the methods outlined by Pharmacia. Human integrin $\alpha_V\beta_3$ was coupled to the surface by injection of 6–9 μl of a 50 ng/μl sample in 10 mM sodium acetate (pH 3.5). Between 5,000 and 10,000 resonance units were immobilized. Subsequently, the sensor chip was deactivated with 1 M ethanolamine (pH 8.5). Binding of Fab to $\alpha_V\beta_3$ was studied by injection of Fab in a range of concentrations (10–600 nM), using PBS as a running buffer. The sensor chip was regenerated with 10 mM HCl and remained active for at least 50 measurements. Based on five measurements at different Fab concentrations, the k_{on} and k_{off} values were calculated using Biacore kinetics evaluation software (Pharmacia) and the equilibrium dissociation constant, K_d, was calculated from k_{off}/k_{on}. The reliability of the data was validated by analyzing the binding of each Fab on at least two different sensor chips and by applying the internal consistency tests suggested by Schuck and Minton (15). In addition, the rough range of the K_d values was independently confirmed by competition ELISA using a procedure described by Friguet *et al.* (16).

RESULTS

cDNA Cloning of LM609. cDNAs encoding the Fd fragment and entire light chain were cloned by PCR from LM609-expressing hybridoma cells (6). The PCR products were cloned into the phage display vector pComb3H (9), which is derived from pComb3 (10), and engineered to facilitate the expression of Fab on the surface of M13 filamentous phage. Phage displaying LM609 Fab were selected by panning against immobilized human integrin $\alpha_V\beta_3$ and the corresponding cDNA sequences were determined. The cloned LM609 Fab purified from *E. coli* demonstrated specific binding to $\alpha_V\beta_3$ using the ELISA.

Humanization of the Light Chain of LM609. Our humanization strategy is outlined in Fig. 1. It involves two selection steps for the sequential humanization of the light chain and the Fd fragment of the heavy chain. Throughout these selections the only preserved sequences in the variable domains of light chain (V_L) and heavy chain (V_H) are two of six CDRs, LCDR3 and HCDR3.

For the humanization of the light chain, the mouse Fd fragment was substituted by a chimeric Fd fragment composed of mouse V_H linked to the human constant domain 1 of the heavy chain, C_H1. A single residue in the mouse FR4 was converted to the corresponding human residue, resulting in complete humanization of the FR4 region. Humanization of the light chain began by substituting the V_κ gene segment of LM609 by a human V_κ and V_λ gene library joined at the LCDR3 junction. As described for the Fd fragment, the FR4 region was humanized by one amino acid change and appended to a human C_κ region. To ensure a highly diverse V gene library, the human antibody sequences were amplified from cDNA prepared from the bone marrow of five healthy individuals using a variety of oligonucleotides that were designed to amplify most of the known human antibody sequences (see *Materials and Methods*). The corresponding phage libraries displaying hybrid Fab were combined and selected by four rounds of panning against immobilized human integrin $\alpha_V\beta_3$. Analysis of the output phage pool from each round for binding to $\alpha_V\beta_3$ by phage ELISA revealed an increasing signal. After the fourth round of selection, six clones that demonstrated strong reactivity to the antigen were studied. DNA sequence analysis of these clones revealed three different light chain sequences. Two light chains (Fig. 2*A*), found in five of six positive clones, differed in only four amino acids (i.e., they were 96% identical), whereas a third light chain sequence shared about 80% identity with the other two (data not shown). This latter sequence consisted of two parts, each of which could be aligned to germ-line genes from different V_κ families; thus, this light chain sequence probably arose from PCR crossover, which has been reported to occur frequently in the amplification of antibody sequences (17).

The selected human light chains are κ light chains as was the original mouse light chain. Databank screening revealed that the selected human light chains are derived from the same germ-line gene, namely, DPK-26, which belongs to the $V_\kappa 6$ family (Fig. 2*A*). This result supports a strong selection for the light chains since the $V_\kappa 6$ family represents only a small fraction of the expressed human V_κ repertoire (18, 19). An obvious reason for this strong selection is the strong sequence similarity between the selected human light chains and the original mouse light chain. Limited sequencing of the unselected library confirmed its diversity and did not reveal any $V_\kappa 6$-containing clones. Moreover, in contrast to the unselected sequences, both LCDR1 and LCDR2 of the selected human light chains are highly similar to the corresponding mouse sequence (Fig. 2*A*). The C-terminal amino acid of FR2 (Kabat position 49; ref. 12) of the original mouse light chain sequence is a lysine, which is an unusual amino acid at this position and, thus, may be involved in the formation of the antigen-binding site. Interestingly, this lysine is conserved in our selected sequences (Fig. 2*A*) as well as in the two known human

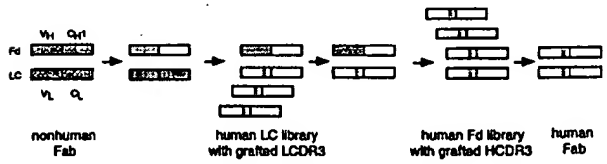

FIG. 1. Humanization of nonhuman monoclonal antibodies by a combination of CDR grafting and V gene shuffling. Nonhuman sequences are shown in gray, human sequences in white. In the first step, a chimeric nonhuman/human Fd fragment is used as a template for the selection of a human light chain that contains the grafted LCDR3 loop of the nonhuman light chain. In the second step, a human Fd fragment that contains the grafted HCDR3 loop of the nonhuman Fd fragment is selected. The sequential V gene shuffling procedure is based on phage display. LCDR3, complementarity-determining region 3 of light chain; HCDR3, complementarity-determining region 3 of heavy chain.

A V_L sequences

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
mouse	ELVMTQTPATLSVTPGDSVSLSC	RASQSISNHLH	WYQQKSHESPRLLIK	YASQSIS	GIPSRFSGSGSGTDFTLSINSVETEDFGMYFC	QQSNSWPHT	FGGGTKLEIK
DPK-26	-I-L--S-DFQ----KEK-TIT-	------GSS--	-----PDQ--K----	-----F-	-V----------T---L-A--AAY-Y-		
human[1]	------S-EFQ----KET-TIT-	----D-GTS--	-----PGQ--K----	----PVF	-V----R----------T-Y-L-A--AV-Y-	----------	--Q-------
human[2]	------S-EFQ----KET-TIT-	----D-G-S--	-----PGQ--K----	----PVF	-V----R----------T-SRL-P---AV-Y-	----------	--Q-------

B V_H sequences

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
mouse	EVQLEESGGGLVKPGGSLKLSCAASGFAFS	SYDMS	WVRQIPEKRLEWVA	KVSSGGGSTYYLDTVQG	RFTISRDNAKNTLYLQMSSLNSEDTAMYYCAR	HNYGSFAY	WGQGTLVTVSA
human[3]	Q---VQ--AEVR---S-VRV--K---GT--	GFAV-	----A-GQ-P--LG	GIVASL---D-AQKF-D	KL--TV-ESTA-VYME-RN-R-D---V-----	--------	----------S
human[4]	Q---Q---P-----SQT-S-T-TV--ASI-	RGGYYMS	-I--Y-GKG---IG	YIH HS-----NPSLKS	-V--AI-TS--Q-S-RLT-VTAA---V-----	--------	----------S
human[5]	Q---Q---P-----SQT-F-T-TV--GSI-	SGGYYWS	-I-HH-GKG---IG	YIH HRAAP--NPSLKS	-V---V-TSR-QIS-KLR-VTAA---V-----	--------	----------S
human[6]	Q---Q---P-----SET-S-T-TV--GSI-	SGGYYMS	-I--H-GKG---IG	YIH HSAG---NPSLKS	-V-M-V-TS--Q-S-KLT-VTAA---V-----	--------	----------S
human[7]	Q---Q---P-----SET-S-T-SV--GSI-	SGGYYMS	-I-HH-GKG---IG	YIH HSAG---NPSLKS	-V-M-A-TS--Q-S-KLA-VTAA---V-----	--------	----------S

FIG. 2. Amino acid sequence alignment of mouse and humanized LM609. Shown are FRs and CDRs. Dashes indicate identical amino acids. Note that due to our grafting procedure CDR3 is identical in the original mouse and the selected human sequences. (*A*) Alignment of the selected human V_L sequences. Databank screening revealed that the two selected human V_L sequences are derived from germ-line DPK-26 of the $V_\kappa 3$ family. (*B*) Alignment of the selected human V_H sequences. Five different human V_H sequences were selected. Four of them, represented by clones 7, 4, 24, and 2, are highly related in amino acid sequence; they share an identical V_L domain and an amino acid sequence identity of at least 85% in their V_H domains. The V_H sequences are all derived from germ-line DP-65 or the highly related DP-78. In contrast, clone 11 represents a humanized version with a V_H domain that is derived from a different germ-line family. This humanized version also contains a different V_L domain which is 96% identical and derived from the same germ-line. Germ-lines were determined by nucleic acid sequence alignment using DNAPLOT software provided by the VBASE Directory of Human V Gene Sequences (http://www.mrc-cpe.cam.ac.uk/imt-doc/). [1]clone 11, germ-line DPK-26 ($V_\kappa 6$ family); [2]clones 2, 4, 7, 24, germ-line DPK-26 ($V_\kappa 6$ family); [3]clone 11, germ-line DP-10 ($V_H 1$ family); [4]clone 7, germ-line DP-78 ($V_H 4$ family); [5]clone 4, germ-line DP-65 ($V_H 4$ family); [6]clone 24, germ-line DP-65 ($V_H 4$ family); [7]clone 2, germ-line DP-65 ($V_H 4$ family).

germ-line $V_\kappa 6$ sequences, whereas all of the unselected sequences that were analyzed contained a tyrosine instead. The $V_\kappa 6$ family is the only human V_κ family that contains a lysine at this position. To study whether the selected human light chains are derived from germ-line V genes that are most similar to the original mouse light chain, the VBASE directory of human V gene sequences (maintained by I. M. Tomlinson, @ http://www.mrc-cpe.cam.ac.uk/imt-doc/) was searched for the highest sequence similarity with the original V_L. Indeed, germ-lines DPK-26 and DPK-25, the only two members of the $V_\kappa 6$ family, were determined to be most similar to the original mouse light chain. Thus, library and databank screening yielded the same result.

Three clones from the light chain selection demonstrated weaker binding to $\alpha_V\beta_3$ than the six clones discussed above, but still gave significant binding above background. DNA sequencing revealed three unrelated V_λ sequences that had no apparent similarity to the original mouse V_κ sequence. The V_λ sequences, along with the selected V_κ sequences (and with the exception of the clone containing the PCR crossover), were used as templates in the humanization of the heavy chain of LM609.

Humanization of the Heavy Chain of LM609. A library of Fd fragments was prepared by stitching human V_H gene libraries onto the chimeric Fd fragment described above. These libraries were paired with the four selected human light chains and were selected by four rounds of panning against immobilized human integrin $\alpha_V\beta_3$. As seen for the human light chain selection, analysis of the output phage pool from each round for binding to $\alpha_V\beta_3$ by phage ELISA revealed an increasing signal. After the fourth round, Fab was produced from single clones and tested for binding to $\alpha_V\beta_3$ by ELISA. Light chain- and Fd fragment-coding sequences from 14 binding clones were determined by DNA sequencing and revealed five different sequences (Fig. 2). The two human κ light chains with the highest sequence homology to the LM609 light chain were reselected. One of them was found to pair with four different Fd fragments that were closely related to each other (85–96% sequence identity) and derived from germ-line DP-65 or DP-78 of the $V_H 4$ family. The other human κ light chain was found to pair with a Fd fragment that was derived from germ-line DP-10 of the $V_H 1$ family. Neither germ-lines from the $V_H 4$ nor the $V_H 1$ family show high sequence homology with the V_H of LM609. Indeed, databank screening yielded a germ-line from the $V_H 3$ family as the best human match for the V_H of LM609. Phylogenetic analysis has shown that $V_H 1$, $V_H 3$, and $V_H 4$ not only form separate families but belong to different clans of the human V_H germ-lines (20). In retrospect, V_λ selections should have been performed independent of V_κ selections to ensure the retention of highly diverse light chains in the humanized antibodies. The V_λ antibodies may have been lost in the selections for reasons other than antibody affinity, such as their relative toxicity to *E. coli*.

Binding Specificity and Affinity of Humanized LM609. Five humanized LM609 versions, represented by clones 11, 7, 4, 24, and 2 were produced as soluble Fab by *E. coli*, purified by affinity chromatography, and their binding specificity and affinity was analyzed. Humanized LM609, which had been selected by binding to immobilized and thus potentially denatured human integrin $\alpha_v\beta_3$, was tested for binding to native human integrin $\alpha_v\beta_3$ expressed on the cell surface. For this, binding of humanized LM609 to untransfected CS-1 hamster cells and CS-1 hamster cells transfected with either human β_3 or β_5 cDNA (7) was analyzed by flow cytometry. By recruiting the endogenous hamster α_v polypeptide, the human β_3 and β_5 polypeptides form functional integrins on the cell surface (7). Like mouse LM609, and in contrast to unrelated human Fab fragments that were used as controls (data not shown), all five humanized versions of LM609 revealed specific binding to CS-1 hamster cells transfected with human β_3 (Fig. 3*A*). Binding of all five humanized LM609 versions to CS-1 cells transfected with human β_3 cDNA could be blocked by an excess of LM609. As shown in Fig. 3*B*, a fourfold molar excess of mouse LM609 IgG blocked binding of humanized LM609 almost completely, whereas the same concentration of mouse

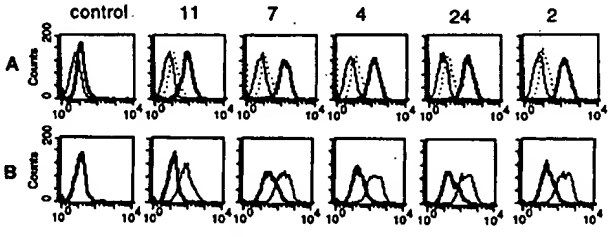

FIG. 3. Flow cytometry histograms demonstrating that humanized LM609 binds specifically to human integrin $\alpha_v\beta_3$ and can be blocked by mouse LM609. (*A*) Binding of humanized LM609 Fab to untransfected CS-1 hamster cells (fine line) and CS-1 hamster cells transfected with either human β_3 (bold line) or β_5 cDNA (dotted line). (*B*) Binding of humanized LM609 Fab to CS-1 hamster cells transfected with human β_3 cDNA in the presence of a fourfold molar excess of mouse AP3 IgG (fine line) or mouse LM609 IgG (bold line). Clones 11, 7, 4, 24, and 2 represent the five humanized LM609 versions (cf. Fig. 2). Controls in *A* and *B* are based on identical experiments using buffer instead of humanized LM609 Fab. The *y* axis gives the number of events in linear scale, the *x* axis the fluorescence intensity in logarithmic scale.

Table 1. Binding kinetics of mouse LM609 and humanized LM609 Fab

Clone	$k_{on}/10^4$, $M^{-1}\,s^{-1}$	$k_{off}/10^{-4}$, s^{-1}	K_d, nM
LM609*	14	4.6	3.3
LM609	8.6	8.6	10
11	1.0	16	160
7	18	5.4	3.0
4	6.8	5.8	8.5
24	13	9.9	7.6
2	11	7.5	6.8

Binding kinetics were determined using surface plasmon resonance. Human integrin $\alpha_v\beta_3$ was immobilized on the sensor chip. The K_d value was calculated from k_{off}/k_{on}. Clones 11, 7, 4, 24, and 2 represent the five humanized LM609 versions (cf. Fig. 2). Fab was produced by *E. coli* except LM609* which was prepared from IgG by papain digestion.

AP3 IgG directed to a different epitope on human integrin $\alpha_v\beta_3$ had no effect. A 20-fold molar excess of LM609 Fab derived from IgG by papain digestion also blocked the binding of humanized LM609 (data not shown). Control experiments revealed that both LM609 and AP3 bound to $\alpha_v\beta_3$ expressed on the cell surface. Potential cross-reactivity of humanized LM609 with human integrin $\alpha_{IIb}\beta_3$ was analyzed by ELISA. Whereas an engineered RGD peptide mimetic antibody Fab-9 with known cross-reactivity (21) bound to both immobilized human integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, cross-reactivity with $\alpha_{IIb}\beta_3$ was not detected for mouse LM609 nor its five humanized versions.

The kinetic parameters of Fab binding to human integrin $\alpha_v\beta_3$ binding were determined using surface plasmon resonance and the affinities were calculated from these kinetic parameters (Table 1). Analysis of these binding data revealed that the four humanized LM609 versions with the high sequence similarity, represented by clones 7, 4, 24, and 2, also show similar affinities for $\alpha_v\beta_3$. Clones 4, 24, and 2, which are derived from the same germ-lines, have almost identical K_d values in the range of 7–9 nM. Clone 7, which shares the same light chain but contains a Fd fragment that is derived from a different, though highly related V_H germ-line, has a moderately higher affinity with a K_d value of 3 nM. In contrast, clone 11 with the unrelated Fd fragment has a much weaker affinity, which is mainly caused by a lower association rate constant (Table 1). LM609 Fab was analyzed for comparison. As Table 1 shows, Fab that was generated by papain digestion from LM609 IgG revealed an affinity with a K_d value of about 3 nM, whereas the affinity of Fab produced by *E. coli* was weaker by a factor of three. It is likely that this discrepancy is partly due to a lower concentration of functional Fab in preparations from *E. coli*. However, the higher dissociation rate constant of *E. coli*-derived LM609 Fab, which is independent from the concentration, indicates that the quality of the antigen-binding site might be affected as well. In any case, the humanized LM609 versions were derived from *E. coli* as well and, thus, should be compared with the corresponding LM609 preparation. Such a comparison (Table 1) shows that four of five humanized antibodies have an affinity that is as good as or better than the original mouse antibody.

DISCUSSION

Though rodent mAbs have long been regarded as powerful therapeutic agents, a major obstacle for clinical applications has been their immunogenicity in humans. Two routes in antibody engineering have been taken to overcome the immunogenicity of mAbs, either the humanization of rodent mAbs or the direct generation of human mAbs. The latter route has recently gained importance with the development of new methodologies that allow the selection of human mAbs from immune, naive, and synthetic human antibody libraries displayed on phage (22, 23) as well as from transgenic mice (24). More than 20 yr of mAb generation by the classical hybridoma technology, however, has yielded a number of promising pharmaceutical candidates and their humanization compares well to the *de novo* generation and characterization of human mAb for accessing clinical applications in the coming years.

Currently, CDR grafting is the most frequently used strategy for the humanization of rodent mAbs (25). In this approach the six CDR loops comprising the antigen-binding site of the rodent mAb are grafted into corresponding human framework regions. CDR grafting takes advantage of the conserved structure of the variable Ig domains, with the four framework regions serving as a scaffold that supports the CDR loops. CDR grafting often yields humanized antibodies with much lower affinity because framework residues are involved in antigen binding, either indirectly, by supporting the conformation of the CDR loops, or directly, by contacting the antigen (26). Therefore, it is usually necessary to replace certain framework residues in addition to CDR grafting. The fact that about 30 framework residues potentially contribute to antigen binding (26) makes this fine-tuning step very laborious. Another humanization strategy is the method of resurfacing (27). In this approach only the surface residues of a rodent antibody are humanized.

Though both CDR grafting and resurfacing are based on rational design strategies and iterative optimization (i.e., site-directed mutagenesis of framework residues aided by computer modeling), selective approaches (i.e., randomization of a small set of framework residues and subsequent selection from phage display libraries) have been reported recently in humanization strategies (28, 29).

In vitro selection and evolution of antibodies derived from phage display libraries have become a powerful tool in antibody engineering (for recent reviews cf. refs. 9 and 30). An entirely selective humanization strategy based on phage display libraries has been reported by Jespers *et al.* (31). In two steps, each polypeptide of the rodent antibody, either light chain or heavy chain, is replaced by a corresponding human polypeptide library and the resulting hybrid antibody library is selected by panning against the particular antigen. Though this strategy may compete with CDR grafting because the arduous fine-tuning steps are unnecessary, the lack of other successful applications of this approach—and our failed attempts to humanize LM609 by this approach for comparative studies—suggest that in contrast to CDR grafting, the general applicability of this approach for antibody humanization is uncertain. Also, since this approach is a sequential chain shuffling procedure, it may lead to the production of a humanized antibody that recognizes a slightly different epitope (32–34). It has been observed that antibodies consisting of the same heavy chain paired with light chains that differ in LCDR3 and elsewhere in V_L may bind different epitopes on the same antigen. It is conceivable that this is an interesting feature in particular cases; however, alteration of antigen specificity following antibody humanization is not desired in general.

Based on these general considerations, we designed a strategy that recognizes the key roles of HCDR3 and LCDR3 in antigen recognition and combined this with a selective approach that eliminates the arduous fine-tuning steps associated with CDR grafting as well as all mouse sequence. This strategy for humanizing antibodies is presented in Fig. 1. In the first step a human light chain library with the grafted original LCDR3 replaces the original light chain and a chimeric Fd fragment consisting of the original V_H Ig domain fused to a human C_H1 Ig domain replaces the original Fd fragment. FR4 of both chains is directly humanized by simple point mutations prior to the first selective step. Sequence changes required in this region should in general be minimal given the substantial homology between mouse and human J genes and should have little effect on the affinity. Human constant regions are preferred to stabilize the hybrid Fab of the first selection step by the interaction of two matching human constant domains C_κ and C_H1. In addition, Fab carrying human constant regions are often better expressed in *E. coli* (35,

36; C.R. and C.F.B. III, unpublished observations), a prerequisite for phage display. In the second step, the selected human light chains from the first step are paired with a human Fd fragment library containing the original HCDR3. Since the heavy chain typically plays the most dominant role in antigen recognition, it is conserved in the first selection step.

By preserving the original LCDR3 and HCDR3 sequences of LM609 while subjecting the remaining sequence to selection, our humanization strategy was designed to ensure antigen specificity and epitope conservation. LCDR3 and HCDR3 contain the hypervariable joints of the V/J and V/D/J gene rearrangements that participate in direct antigen contact in all studied antigen/antibody complexes (37). Unlike the other CDR regions, both LCDR3 and HCDR3 interact with all three CDRs of the other variable domain (38). Thus, although generalizations might be misleading (37), LCDR3 and HCDR3 can be considered to make the most significant contributions to affinity and specificity. Given the tremendous sequence diversity displayed by human antibodies in these regions and the mechanism of its generation, it is difficult, if not impossible in most cases, to classify sequences of these regions as either mouse or human. Thus, from the perspective of sequence these antibodies may be considered completely human. Human HCDR3s are, however, on average longer than mouse HCDR3s and encompass the full range of lengths utilized by mice (39). The HCDR3 length of eight amino acids found in LM609 is well represented in both mouse and human antibodies (39). HCDR3 length will not likely be a significant issue in mouse to human conversions.

Antigen specificity and epitope conservation are critical demands in the humanization of LM609. LM609 binds to a conformational epitope on human integrin $\alpha_v\beta_3$. Importantly, by binding to this epitope LM609 induces apoptosis in vascular cells expressing $\alpha_v\beta_3$ (3). In contrast to antibodies that are engineered RGD peptide mimics (21), LM609 does not recognize the related human integrin $\alpha_{IIb}\beta_3$. A cross-reactivity with human integrin $\alpha_{IIb}\beta_3$, which is expressed on platelets, would preclude the use of LM609 as a tool in cancer therapy. The five humanized LM609 versions were analyzed for antigen specificity in terms of cross-reactivity with the human integrins $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_5$, which are closely related to $\alpha_V\beta_3$ in sequence and function (40). Neither $\alpha_{IIb}\beta_3$ nor $\alpha_V\beta_5$ was recognized by the five humanized versions of LM609. In addition, all appeared to bind to the same epitope on $\alpha_V\beta_3$ as LM609, as their binding was specifically blocked in the presence of a molar excess of LM609 but not of AP3, a mAb that binds a different epitope on human integrin $\alpha_v\beta_3$. In addition to the binding specificity, the binding affinity provides evidence that epitope conservation was obtained; three of five humanized LM609 versions bound $\alpha_v\beta_3$ with an affinity very similar to that of LM609. Yet, the contribution of the selected CDRs to the antigen-binding site is obvious from the fact that one humanized LM609 version binds $\alpha_v\beta_3$ with higher, and another with slightly lower, affinity.

The generation of different humanized versions of the parental antibody is an attractive result of this methodology. CDR grafting, in contrast, generates a single humanized version. It is anticipated that an antiangiogenic strategy for cancer therapy would require long-term administration of antibody. It is conceivable that repeated therapeutic application may produce an antiidiotypic response that ablates the efficacy of the antibody. Administration of an equally potent antibody that is unreactive to the antiidiotypic response generated by the first would allow therapy to continue. Indeed, introduction of modest changes within the variable domain of an antibody can dramatically alter its reactivity to an antiidiotypic response (41). Finally, a practical advantage of producing multiple humanized antibodies is that the expression level of antibodies is antibody dependent. Thus, one or more of the humanized antibodies may be more suitable for large-scale production.

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